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Keywords (separated by '-')	TIRFM - Evanescent field - Exocytotic events - SNARE proteins - MSD - Diffusion coefficient - Chromaffin granules - Chromaffin cells	

Chapter 25

Studies of the Secretory Machinery Dynamics by Total Internal Reflection Fluorescence Microscopy in Bovine Adrenal Chromaffin Cells

José Villanueva, Yolanda Gimenez-Molina, and Luis M. Gutiérrez

Abstract

Cultured bovine chromaffin cells have been tested as a successful neuroendocrine model to study the secretory process. Changes in the dynamics of the secretory vesicles and the exocytotic machinery microdomains could be studied in control and stimulated conditions using appropriate molecular tools such as fluorescent SNARE protein expression or fluorochrome vesicular labeling in these neuroendocrine cells. Since most of these changes occur in or near the plasma membrane, the use of the total internal reflection fluorescent microscopy (TIRFM) and the implement of particle motion analysis could be essential tools to study the structural and dynamic changes of secretory machinery related with its function in this exocytotic cell model.

Key words TIRFM, Evanescent field, Exocytotic events, SNARE proteins, MSD, Diffusion coefficient, Chromaffin granules, Chromaffin cells

1 Introduction

Neurosecretion involves the transport of specialized vesicles to the immediate vicinity of active sites and they regulated fusion. In this sense, the initial transport of the vesicles is governed by the activity of the cell cytoskeleton [1] whereas SNARE proteins appear to be essential for the final exocytotic fusion steps [2]. Since these final stages of neurosecretion occur nearby the plasma membrane, the study of these structural and dynamic changes requires microscopic techniques with a good signal/noise ratio in the *z*-axis perpendicular to the plasma membrane plane. Confocal techniques have a relatively good *z*-axis resolution but the laser excitation field extends up to ~600 nm generating an elevated *z*-axis background [3]. On the other hand, in TIRFM techniques the excitation field, named evanescent wave, is thinner extending around ~200 nm in the interior of the cells attached to the coverslip surface (Fig. 1) (*see* **Note 1**) [4], and in consequence only fluorophore molecules

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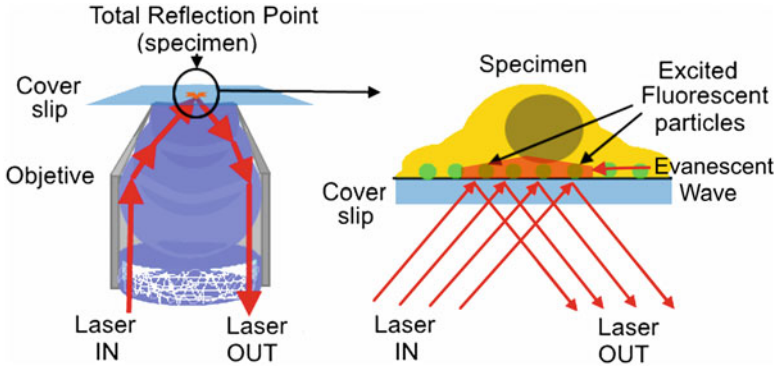

Fig. 1 Total internal reflection microscopy. When laser light (IN) strikes the interface between two optical media of different refractive indices (i.e., objective/specimen), at an angle greater than the critical angle undergoes total reflection. Beyond the angle of total reflection, the electromagnetic field (termed *evanescent wave*) still extends a few hundred nanometers into the *z* direction but decreasing exponentially with the distance, therefore exciting fluorescent molecules within this evanescent field (*see* **Note 4**)

located in the surface of the specimen are excited to emit. In other words, TIRFM makes possible to obtain a high contrast observation of the secretory machinery behavior in or near the plasma membrane having a good signal/noise ratio [5, 6].

The employment of TIRFM techniques for analyzing dynamic changes in SNARE fluorescent constructs (i.e., GFP-SNAP-25) expressed in chromaffin cells [7] has been demonstrated previously to be a useful tool for studying both the fusion complex assembly and the vesicle fusion kinetics [4]. In the same way, the use of fluorescent markers for acidic chromaffin granules has allowed to design and perform dynamic TIRFM assays to analyze both the mobility and fusion parameters changes in response to different secretory conditions [4, 8, 9].

This work describes the methodological approach in order to carry out these studies in bovine chromaffin cells in monolayer cultures.

2 Materials

2.1 Basal and Depolarizing Solutions

All solutions have to be prepared using ultrapure water (purifying deionized water, sensitivity of 18 MΩ-cm at 25 °C and analytical grade reagents). Prepare and store all reagents at 4 °C (unless indicated otherwise).

1. Krebs/HEPES (K/H) basal solution (134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$,

11 mM glucose, 0.56 mM ascorbic acid, and 15 mM HEPES, pH 7.4).

2. Krebs/HEPES (High K) depolarizing solution (80 mM NaCl, 59 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11 mM glucose, 0.56 mM ascorbic acid, and 15 mM HEPES, pH 7.4).

2.2 Regulation for the Use of Bovine Adrenal Glands

Bovine adrenal glands were obtained from an industrial slaughterhouse that is subject to strict regulations issued by the Spanish Ministries of Agriculture, Industry and Health, and in accordance with European Community guidelines.

All the protocols described here were approved by the "Organo Evaluador de Proyecto" at the University Miguel Hernández, the office in charge of overseeing the ethical issues associated with animal care and experimentation at our investigation institute.

2.3 Chromaffin Cell Reagents, Solutions, and Fungibles

1. Locke buffer 1× (154 mM NaCl, 5.58 mM KCl, 3.6 mM $NaHCO_3$, 5.6 mM glucose, 5 mM HEPES, sterilized, adjusted to pH 7.4, and stored at 4 °C).
2. Type A Collagenase (store at 4 °C).
3. Bovine serum albumin (BSA) (store at 4 °C).
4. Type A Collagenase Solution (Cold Locke buffer 1×, 0.25% Type A Collagenase, 0.5 % BSA) should be prepared before use.
5. Percoll continuous gradient (store at 4 °C).
6. Diluted Percoll Continuous Gradient (mix fresh 36 mL of Percoll Continuous Gradient with 4 mL of Locke buffer 1×, maintaining at 37 °C until use).
7. Dulbecco's modified Eagle's medium (DMEM) (store at 4 °C).
8. Supplemented DMEM (DMEM supplemented with 10% fetal calf serum, 10 μM cytosine arabinoside, 10 μM 5-fluoro-2′-deoxyuridine, 50 IU/mL penicillin, and 50 μg/mL streptomycin, stored at 4 °C).
9. 35 mm Petri dishes.

2.4 Fluorescent Protein Expression

The use of expression vectors is a very useful tool for dynamic studies using fluorescence microscopy. In this way, we used the GFP-SNAP-25 construct like an example to analyze SNARE protein behavior. This construct was obtained by cloning the cDNA corresponding to the SNAP-25a isoform [10] into the XhoI and BamHI sites of pEGFP-C3 expression vector (Clontech, Palo Alto, CA). This construct expresses the protein SNAP-25 fused in-frame at the C-terminus to EGFP.

2.5 Fluorescent Beads, Fluorochrome Dyes, and Working Solutions

1. 100 nm Fluorescent beads.
2. Lyso Red Stock Solution: Lysotracker Red DND-99 (Invitrogen). Resuspend at 1 mM in DMSO.
3. Acridine Orange Stock Solution: Acridine Orange.
4. Lyso Green Stock Solution: LysoTracker Green DND-26 (Invitrogen): Resuspend at 1 mM in DMSO.
5. Prepare Lyso Red or Lyso Green Working Solutions (1 μM) by adding 1 μL from each stock solution to a 1 mL of Krebs/HEPES (K/H) basal solution.
6. Prepare Acridine Orange Working Solution (2 μM) by adding 2 μL from Acridine Orange Stock Solution to a 1 mL of Krebs/HEPES (K/H) basal solution.

2.6 DNA Electroporation Kit

Amaxa basic nucleofector kit for primary mammalian neuronal cells (Amaxa GmbH).

2.7 TIRFM Microscopy

1. A through-the-lens TIRFM system using an Olympus IX-71 inverted microscope with a 100× PlanApo oil immersion TIRFM objective with 1.45 N.A. (numeric aperture).
2. Epifluorescence and laser illumination (488 nm argon ion 40 mW or 543 nm He/Ne 10 mW) using an Olympus TIRFM IX2-RFAEVA combiner system that allows changing of the angle of laser incidence.
3. Fluorescence emission is split using an Optosplit II system (Cairn Research Ltd.) equipped with GFP and rhodamine filter sets.
4. Acquire separated images simultaneously using an Electron Multiplier CCD cooled camera (Hamamatsu), controlled by the Imaging Software Wasabi v.1.5 (Hamamatsu Photonics) in an IBM-compatible PC.

2.8 MSD Algorithm and Analysis Software

1. Use the public domain ImageJ program with plug-ins to analyze the fluorescent images.
2. After thresholding of the images, determine particle centroid in time-lapse studies using a multi-tracker plug-in as described earlier [3, 11, 12].
3. Transform the *x*–*y* coordinates for centroids of these particles to Igor Pro program (WaveMetrics Inc.), and use specialized macros to calculate the total lateral displacement and the mean square displacement (MSD) for any given time interval by using Eqs. (1) and (2) (*see* **Notes 1** and **2**).

3 Methods

3.1 Isolation, Culture, and Transfection of Bovine Chromaffin Cells

1. Prepare bovine chromaffin cells by collagenase digestion and separate from debris and blood cells using a centrifugation in Percoll gradients as described before [13].
2. Store adrenal glands in cold (4 °C) Locke buffer.
3. Trim each gland of fat and perfuse, using a syringe, via adrenal vein with 3–5 mL of 37 °C warned Type A Collagenase Solution over a period of 15 min while maintained at 37 °C. Repeat up to three times.
4. Dissect each gland using scissors and mechanically scrape and disaggregate the exposed medulla to obtain a cell homogenate.
5. Resuspend the resulting solution with warmed (37 °C) Locke buffer and filter successively using first a sieve of 217 micrometer diameter and a second one of 82 μm diameter.
6. Add the resulting cell suspension to a Locke buffer to a final volume of 200 mL and centrifuge ($100 \times g$ for 5 min) in Locke buffer at room temperature to wash out collagenase.
7. Resuspend cell pellets using Locke buffer up to a final volume of 42 mL and mix with 38 mL of diluted Percoll continuous gradient.
8. Centrifuge for 22 min at 37 °C in a Beckman JA 25.50 rotor at $21{,}000 \times g$.
9. Collect cells equilibrated between the densities of 1.045 and 1.075 g/mL. This gradient fraction contains chromaffin cells without blood cells and debris.
10. Wash the isolated chromaffin cells twice by centrifugation ($100 \times g$ for 5 min) in Locke buffer at room temperature.
11. Harvest isolated chromaffin cells and resuspend with 10 mL of Dulbecco's modified Eagle's medium (DMEM).
12. Count cells in a Neubauer chamber to estimate cell number and concentration.
13. To obtain culture cells, plate aliquots containing 750,000 isolated chromaffin cells in 35 mm Petri dishes as monolayer cultures with supplemented DMEM (150,000 cells/cm^2), and transferred to an incubator 37 °C 5% CO_2. These cells could be used for experimentation during a week.
14. Transfect aliquots containing five million isolated chromaffin cells with 1–3 μg of the appropriate DNA expression plasmid (GFP-SNAP-25) using the Amaxa basic nucleofector kit for primary mammalian neuronal cells according to the manufacturer's instructions.

15. Plate transfected cells in 35 mm Petri dishes as monolayer cultures with supplemented DMEM (150,000 cells/cm^2), and maintained in a temperature incubator as described before (*see* **step 4**). Use the transfected cells between the second and fourth days after plating.

3.2 Fluorochrome Vesicle Labeling

1. Replace the culture media in each 35 mm Petri dish with Krebs/HEPES (K/H) basal solution.
2. To stain a population of acidic vesicles for experiments involving the motion of granules incubate cells for 20 min at room temperature and darkness with either Lyso Red or Lyso Green Working solution (1 μM) [14].
3. For experiments aiming to measure both vesicular motion and fusion incubate cells for 20 min at room temperature and darkness with Acridine Orange Working Solution (2 μM) [4].
4. Wash cells extensively with Krebs/HEPES (K/H) basal solution and mount to use them for TIRFM experiments within the next 2 h.

3.3 TIRFM Laser Calibration and Alignment

1. Calibrate TIRFM using 100 nm fluorescent beads adhered to the coverslip.
2. Determine the fluorescence intensities of the beads at different vertical planes with step sizes of 100 nm using the motorized system mounted on the microscope. Obtain images for both epifluorescence and TIRFM.
3. Estimate the depth of penetration for the evanescent field to ~200 nm ($d = 180 \pm 16$ nm) (*see* **Note 5**) to permit visualization of the static beads adhered to the coverslip. Beads in suspension undergoing random movement should be infrequently seen in TIRFM, and the vast majority visualized by epifluorescence.

3.4 TIRFM Dynamic Assays

1. For vesicle motion assays, label cells with red acridine orange fluorescence or by LysoTracker red fluorescence (*see* Subheading 3.2). Visualize the cells using a laser excitation 543 nm He/Ne 10 mW [9].
2. For SNARE position, use cells expressing GFP-SNAP-25 (*see* Subheading 3.1). Visualize cells using laser excitation 488 nm argon ion 40 mW [4, 9].
3. Wash cells with Krebs/HEPES (K/H) basal solution for control conditions or treat with different substances for a variety of experimental conditions. After extensive washing, observe the cells under TIRFM using the appropriate laser excitation (543 nm He/Ne 10 mW for labeled vesicles or 488 nm argon ion 40 mW for GFP-SNAP25). Acquire fluorescence images at 1-s intervals during 20 s.

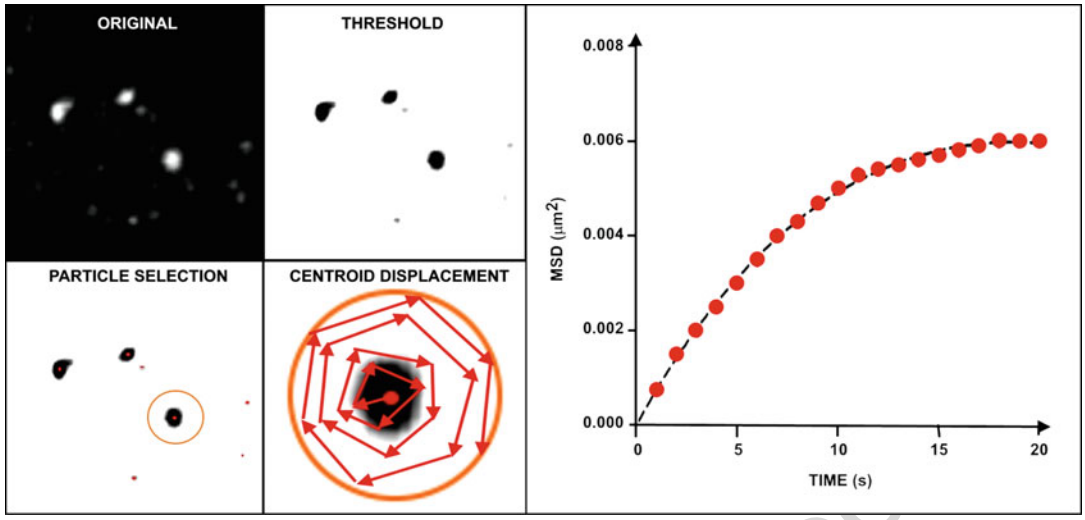

Fig. 2 TIRFM dynamic assays. Time-lapse original fluorescent images were processed and imported in ImageJ program to select particles and measure centroid *XY* displacement (*see* Subheading 2.4). *XY* coordinates for centroids were imported and processed with specialized macros in Igor Pro program to calculate the mean square displacement (MSD) and the diffusion parameters for any given time interval [15] (*see* **Note 6**)

4. For the motion analysis, threshold fluorescent images to determined particle centroid in time-lapse studies using a plug-in in Igor Pro program as described earlier. Analyze trajectories to calculate average speeds, in each assay conditions.
5. Use specialized macros in Igor Pro program *xy* coordinates for centroids to calculate the total lateral displacement and the mean square displacement (MSD).
6. Export MSD data to Prism Graph Pad v.4.01 software to represent the MSD curves (Fig. 2) [15] (*see* **Note 6**). In each condition of assay, express the averaged MSD data from a number (n) of individual cells from at least three different cultures as a mean ± S.E.M.
7. Calculate the diffusion coefficient D (*see* **Note 2**, Eq. (2)) in some assays assuming that movement was governed by a single coefficient of diffusion and its value could be derived from the fitted slope of the lineal regression for averaged MSD data: $D = \text{slope}/4$ [4, 16].
8. In other assays, fit MSD-averaged data to a nonlinear regression using a hyperbolic equation to determine whether displacement of the particles could be restricted to an area (Fig. 2) [8].
9. Establish the statistical significance of changes in experimental data between different assay conditions using the Student's t-test for paired samples or the two-way ANOVA test (behaviors were considered significantly different when $P < 0.05$).

3.5 TIRFM Fusion Assays

1. For vesicle fusion assays, using acridine orange to label granules (*see* Subheading 3.2). Assess the granules by the red acridine orange fluorescence in mature acidic vesicles whereas their fusion is followed by the green flashes produced after granule matrix neutralization during exocytosis according to procedures described previously [4] or by the total or partial disappearance of red acridine orange fluorescence after exocytosis (Fig. 3).
2. Stimulate cells for 1 min using cell perfusion with Krebs/HEPES (High K) depolarizing solution.
3. In these experiments, keep the laser intensity low (2–4% of the maximal intensity with the 488 nm argon ion 40 mW laser) to prevent light-induced fusion [17]. No fusion should be detected in the absence of cell stimulation.
4. For the analysis of acridine orange-labeled vesicle fusion take images in the green channel at 20-ms intervals. Subject fusion flashes to maximal intensity determination and the data transferred to Igor Pro.
5. Analyze fusion events using software developed for amperometric detection of exocytotic events (Quanta analysis [18]). Obtain the kinetic parameters such as the time at the half-height amplitude ($t_{1/2}$) for hundreds of fusion events and represent distributions using Prism Graph Pad v.4.01 software.

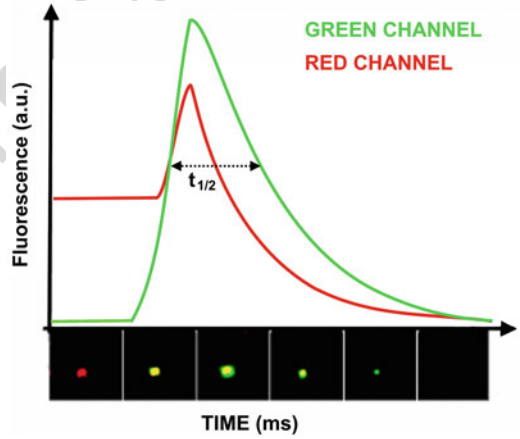

Fig. 3 TIRFM fusion assays. Using a perfusion system, cells were stimulated for 1 min using a depolarizing solution with 59 mM high potassium. Green channel images showed transitory fusion flashes and red channel images showed a disappearing of previous emission. Both registers were subjected to intensity determination and transferred to Igor Pro. Fusion events were analyzed using software developed for amperometric detection of exocytotic events (Quanta analysis [18]). Kinetic parameters such as the time at the half-height amplitude ($t_{1/2}$) were obtained for hundreds of fusion events in each condition assayed

Average the fusion peak shape aged for individual cells and examine for statistical variations (*see* Subheading 3.5, **step 9**). Perform all the functional experiments at 21–22 °C.

4 Notes

1. Equation (1)

$$\text{MSD}\,(n\delta t) = \frac{1}{(N-n)} \sum_{j=1}^{N-n} \left\{ [x(j\delta t + n\delta t) - x(j\delta t)]^2 + [y(j\delta t + n\delta t) - y(j\delta t)]^2 \right\} \quad (1)$$

where δt are the intervals in which images were taken. The coordinates of a centroid were $x(j\delta t)$ and $y(j\delta t)$ for one image and $x(j\delta t + n\delta t)$ and $y(j\delta t + n\delta t)$ for the other image. The displacement during the interval $n\delta t$ can be measured for $(N - n)$ intervals in each temporal sequence. N is the total number of images acquired.

2. Equation (2)

$$\text{MSD}\,(\Delta t) = r_c^2 \left[1 - A_1 \exp\left(\frac{-4A_2 D \Delta t}{r_c^2} \right) \right] \quad (2)$$

The first two terms in an infinite series are represented by this equation. A_1 and A_2 are constants (0.99 and 0.85, respectively), D is the coefficient of confined diffusion, and r_c is the radius of the theoretical circular cage. MSD could be used to calculate r_c when $\Delta t \to \infty$ and the data reach an asymptotic value.

3. Total reflection in the interface between two optical media with different refractive indexes could be described by the following Eq. (3):

$$(\theta c) = \sin^{-1}(n(2)/n(1)) \quad (3)$$

where $n(1) > n(2)$, $n(1)$ is the refractive index of the objective, $n(2)$ is the refractive index of the specimen, and $\theta(c)$ is the critical angle.

4. The evanescent wave created by total reflection extends in the z-axis but it decreases exponentially with the distance following this Eq. (4):

$$I = I_{(0)}\, e^{-z/d} \quad \text{or } z = -d \ \ln\left(I/I_{(0)}\right) \quad (4)$$

5. I is the maximum fluorescence of a particle, $I_{(0)}$ is 255 (maximum of fluorescence for 8-bit images), z is the distance from the interphase of total reflection, and d is the constant of

exponential decay of the evanescent wave that depends on the specific properties of the TIRFM equipment (i.e., numeric aperture of the objective, etc.), by Eq. (5):

$$d = \frac{\lambda}{4\pi}\left(n_2^2\,\mathrm{sen}^2\theta - n_1^2\right)^{1/2} \quad (5)$$

In our system, we calculated $d = 180 \pm 16$ nm and consequently we can excite and see particles nearby plasmatic membrane until approximately 200 nm inside [4].

6. TIRFM dynamic assays can be performed using either fluorescence SNARE protein constructs or fluorescent vesicular markers. The protocol for these studies was very similar (*see* Subheading 3.4): time series TIRFM images were acquired at 1-s intervals during 20 s. The images were stored in a 16-bit HIS format, and exported in 8-bit TIFF multi-file format. Multi-files were then imported to ImageJ program and converted to an 8-bit TIFF stack time-lapse file. This file was processed using threshold option to assess the position of individual particles. The degree of threshold was specific for each type of fluorescent dye. The measurement settings were selected to find the centroid XY position for every particle studied. Black-and-white resulting images were processed with a multitracker plug-in (https://imagej.nih.gov/ij/plugins/multitracker.html). The minimum and maximum size parameters for particle selection and tracking were selected in base to the reported sizes found in bibliography [4, 16, 19]. Individual XY particle displacement data were copied to exported to Igor Pro datasheets. Then data were processed with the MSD macro based on MSD algorithm. Finally, MSD data were copied to export to Prism Graph Pad software v.4.01 to represent the MSD curves (Fig. 2).

Acknowledgments

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